

**ESS Method 150.1:
Chlorophyll - Spectrophotometric**

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Revised September 1991

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1.0 Application

- 1.1 Chlorophyll *a*, a characteristic algal pigment, constitutes approximately 1% to 2% (dry weight) of planktonic algal biomass. This feature makes chlorophyll *a* a convenient indicator of algal biomass.
- 1.2 This method is applicable to most surface waters.

2.0 Summary of Method

- 2.1 Algal cells are concentrated by filtering a known volume of water through a membrane filter (47 mm, 5.0 μm pore size). The pigments are extracted from the concentrated algal sample in an aqueous solution of acetone. The chlorophyll *a* concentration is determined spectrophotometrically by measuring the absorbance (optical density - OD) of the extract at various wavelengths. The resulting absorbance measurements are then applied to a standard equation.

3.0 Sample Preservation and Preparation

- 3.1 Chlorophyll *a* samples should be placed in a dark cooler and packed in ice at the time of collection.
- 3.2 Filter from 50 to 2000 mL of sample through a 5 μm membrane filter, applying vacuum until the sample is dry.
 - 3.2.1 Add 0.2 mL of MgCO_3 suspension during the final phase of the filtration.
 - 3.2.2 Fold the filter into quarters, wrap in aluminum foil; place in a desiccator and freeze.
 - 3.2.3 Samples may be held frozen for up to 30 days if taken from waters of pH 7 or greater. Samples from acidic waters should be processed promptly.

4.0 Comments

- 4.1 Pheophytin, a natural degradation product of chlorophyll, has an absorption peak in the same spectral region as chlorophyll *a*. It may be necessary to make a correction when pheophytin concentration becomes significantly high.
 - 4.1.1 Corrected chlorophyll *a* refers to the method with the pheophytin correction (acidification method).
 - 4.1.2 Uncorrected chlorophyll *a* refers to the method without the pheophytin correction (Trichromatic method).

- 4.2 Handle samples in subdued light to prevent photochemical breakdown of the chlorophyll.
- 4.3 Protect the acetone extract from more than momentary exposure to light.

5.0 Apparatus

- 5.1 Sonicator cell disruptor, Heat Systems-Ultrasonics Inc., Model W-220F, equipped with a microtip.
- 5.2 Beckman Model DU-6 scanning spectrophotometer, 2.0 nm slit or narrower.
 - 5.2.1 Printer.
 - 5.2.2 1.0, 5.0, 10.0 cm spectrophotometer cells.
- 5.3 Calibrated 15 mL centrifuge tubes with teflon lined caps.
- 5.4 Centrifuge capable of attaining 500 g.
- 5.5 Dark box: Light tight box capable of holding a small test tube rack.
- 5.6 Standard laboratory glassware including membrane filtration apparatus.
- 5.7 Millipore SM 5.0 μm membrane filters (47 mm).
- 5.8 Vacuum source.

6.0 Reagents

- 6.1 Aqueous acetone solution: Mix 90 parts reagent grade acetone with 10 parts Milli-Q water (Millipore Reagent Grade Water System).
- 6.2 0.1 N Hydrochloric acid: Add 8.3 mL of reagent grade hydrochloric acid and dilute to 1000 mL with Milli-Q water.
- 6.3 1% Magnesium carbonate suspension: Add 1.0 g of magnesium carbonate powder to 100 mL of Milli-Q water.

7.0 Procedure

- 7.1 Place the filter containing the concentrated algal sample in a centrifuge tube.
 - 7.1.1 Add about 10 mL of aqueous acetone solution; cap tightly and place in the dark box.
- 7.2 Repeat Step 7.1 until the desired number of samples have been processed.

- 7.3 Remove the cap from the centrifuge tube, insert the microtip, and sonify for 20 seconds at the 5 setting.
- 7.3.1 Rinse the microtip into the centrifuge tube with approximately 1 mL of aqueous acetone solution.
- 7.3.2 Bring the extract to a volume of 13.0 mL with the acetone solution, cap, mix and return to the dark box.
- 7.3.3 Repeat the steps outlined in Step 7.3 until all of the samples have been sonified.
- 7.4 Place the dark box in the 4°C cold room and allow the extract to steep overnight.
- 7.5 Clarify the extract by centrifuging the extract for 20 minutes at approximately 500 g. (Mix the extract thoroughly before centrifuging.)
- 7.6 Carefully transfer the clear extract to a 5.0 cm cell and using the multi wavelength mode on the spectrophotometer, measure the absorbance at: 750, 663, 645, and 630 nm (if uncorrected chlorophyll is desired) or at 750, 665, 663, 645, and 630 nm if both corrected and uncorrected chlorophyll are desired).
- 7.6.1 Use a shorter or longer cell as necessary to maintain absorbance between approximately 0.1-1.0 at 663 nm.
- 7.6.2 Note: Operate the spectrophotometer at a slit width no wider than 2 nm for maximum resolution.
- 7.7 For corrected samples: Immediately after measuring the absorbance, add 0.1 mL of 0.1 N HCl to the spectrophotometer cell, mix, wait 90 seconds and measure the absorbance specified in Step 7.6.
- 7.8 Discard the sample, rinse the cell two times with 5 mL portions of aqueous acetone solution.
- 7.9 Repeat Steps 7.6 through 7.8 until all of the samples have been measured.

8.0 Calculations (manual)

- 8.1 Determine the absorbance at 750, 663, 645, and 630 nm directly from the printout.
- 8.2 Subtract the absorbance at 750 nm from the 630, 645, and 663 nm values (turbidity correction).

- 8.3 Calculate the uncorrected chlorophyll *a* concentration by inserting the corrected absorbance values in the following equation.

$$\text{Uncorrected Chlorophyll } a \text{ } (\mu\text{g/L}) = \frac{[11.64 (\text{Abs}663) - 2.16 (\text{Abs}645) + 0.10 (\text{Abs}630)] E(F)}{V(L)}$$

Where *F* = Dilution Factor (i.e., if the 663 Abs is >0.99 with the 1 cm cell, dilute, re-analyze and insert the dilution factor in the equation)

E = The volume of acetone used for the extraction (mL)

V = The volume of water filtered (L)

L = The cell path length (cm)

- 8.4 For corrected samples, determine the absorbance at 665 nm and 750 nm after acidification.
- 8.5 Subtract the absorbance at 750 nm from the absorbance at 665 nm (turbidity correction).
- 8.6 Calculate the corrected chlorophyll *a* and Pheophytin *a* concentration by inserting the turbidity corrected absorbance readings in the following equations.

$$\text{Corrected Chlorophyll } a \text{ } (\mu\text{g/l}) = \frac{26.73(663_b - 665_a) E(F)}{V(L)}$$

$$\text{Pheophytin } a \text{ } (\mu\text{g/l}) = \frac{26.73(1.7 \times [665_a] - 663_b) E(F)}{V(L)}$$

Where *F* = Dilution Factor (if the extract requires dilution)

E = The volume of acetone used for the extraction (mL)

V = The volume of water filtered (L)

L = The cell path length (cm)

665_a = The turbidity corrected Abs at 665 nm after acidification

663_b = The turbidity corrected Abs at 663 nm before acidification

9.0 Computer Automated Calculations

- 9.1 A personal computer may be used to calculate the chlorophyll concentrations, and to evaluate the process quality control data. This technique greatly increases the speed of the analyses and significantly reduces computational and transcription errors.
- 9.1.1 Detailed, step by step instructions for the computer automated method are available in the PC Laboratory Automation Manual.

- 9.2 Equipment needed
 - 9.2.1 IBM or IBM compatible PC with an RS232C board.
 - 9.2.2 Beckman DU-6 communication software or other data acquisition software (Procomm, Lotus (Lotus measure), Measure, etc.).
 - 9.2.3 Spreadsheet software; Lotus 1,2,3.
- 9.3 Data transfer
 - 9.3.1 Connect the PC to the Beckman DU-6 via the RS232 ports.
 - 9.3.2 Install the Beckman data capture software and select the appropriate setup from the main menu. Create a file for the transferred absorbance values.
 - 9.3.3 On the spectrophotometer, select 'output' from 'Data I/O'. Run the samples with the appropriate wavelengths.
 - 9.3.4 After completing the analyses, transfer the file containing the absorbance data to a Lotus 1,2,3 spreadsheet (programmed to perform the calculations in Section 8.0) to calculate the final chlorophyll results.
 - 9.3.5 Evaluate the quality control data and print a final report for subsequent review by another qualified analyst.
 - 9.3.6 After the data review, transfer the result to the Laboratory Information System (LIMS) database.

10.0 Precision and Accuracy

Precision and accuracy data are available in the Inorganic Chemistry Quality Assurance manual.

11.0 References

- 11.1 Biological Field and Laboratory Methods for the Quality of Surface Waters and Effluents, U.S. Environmental Protection Agency, EPA-670/4-73-001, p. 14, (1973).
- 11.2 Nelson, D.H., "Improved Chlorophyll Extraction Method", Science, 132, p. 351, (1960).
- 11.3 Rai, H., "Methods Involving the Determination of Photosynthetic Pigments using Spectrophotometry", Verh. Internat. Verein. Limnol. 18, pp. 1864-1875, (1973).
- 11.4 Standard Methods for the Examination of Water and Wastewater, 14th Ed. pp. 1029-1033, (1975).
- 11.5 Standard Methods for the Examination of Water and Wastewater, 17th Ed. pp. 10-31-10-39, (1989).